Dopamine β-Hydroxylase-Deficient Mice Have Normal Densities of D_2 Dopamine Receptors in the High-Affinity State Based on In Vivo PET Imaging and In Vitro Radioligand Binding

METTE SKINBJERG,[1,2,3] NICHOLAS SENECA,[1] JEIH-SAN LIOW,[1] JINSOO HONG,[1] DAVID WEINSHENKER,[4] VICTOR W. PIKE,[1] CHRISTER HALLDIN,[2] DAVID R. SIBLEY,[3] AND ROBERT B. INNIS[1]*

[1]*Molecular Imaging Branch, National Institute of Mental Health, Bethesda, Maryland*
[2]*Department of Clinical Neuroscience, Psychiatry Section, Karolinska Institutet, Stockholm, Sweden*
[3]*Molecular Neuropharmacology Section, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland*
[4]*Department of Human Genetics, Emory University, Atlanta, Georgia*

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ABSTRACT In vitro, D_2 dopamine receptors (DAR) can exist in low- and high-affinity states for agonists and increases of D_2 receptors in high-affinity state have been proposed to underlie DA receptor supersensitivity in vivo. Deletion of the gene for dopamine β-hydroxylase (DBH) causes mice to become hypersensitive to the effects of psychostimulants, and in vitro radioligand binding results suggest an increased percentage of D_2 receptors in a high-affinity state. To determine whether DBH knockout mice display an increase of high-affinity state D_2 receptors in vivo, we scanned DBH knockout and control mice with the agonist PET radioligand [^{11}C]MNPA, which is thought to bind preferentially to the high-affinity state of the D_2 receptor. In addition, we performed in vitro binding experiments on striatal homogenates with [^{3}H]methylspiperone to measure B_{max} values and the percentages of high- and low-affinity states of the D_2 receptor. We found that the in vivo striatal binding of [^{11}C]MNPA was similar in DBH knockout mice and heterozygous controls and the in vitro B_{max} values and percentages of D_2 receptors in the high-affinity state, were not significantly different between these two groups. In summary, our results suggest that DBH knockout mice have normal levels of D_2 receptors in the high-affinity state and that additional mechanisms contribute to their behavioral sensitivity to psychostimulants. **Synapse 00:000–000, 2010.**

INTRODUCTION

The D_2 dopamine receptor exists in two affinity states for agonists in vitro: a low-affinity state and a high-affinity state that reflects an active form of the receptor that is competent for signaling (Sibley et al., 1982). An increase in the ability of agonists to promote the high-affinity coupling state of the D_2 receptor has been proposed to underlie behavioral D_2 supersensitivity in several animal models such as unilaterally 6-OHDA-lesioned and amphetamine-sensitized rats (Seeman et al., 2002, 2005), but until recently there has been no way to assess an increase in the high-affinity state of D_2 receptors in vivo. In theory, a full D_2 receptor agonist radioligand should selectively label high-affinity state receptors in vivo. Notably, one such compound, [^{11}C]-(+)-PHNO, failed to detect an increase in high-affinity state D_2 receptors in rats

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*Correspondence to: Robert Innis, Molecular Imaging Branch, National Institute of Mental Health, 31 Center Drive, Bethesda, MD 20892-2035. E-mail: robert.innis@nih.gov

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with behavioral D_2 hypersensitivity (McCormick et al., 2009).

A common polymorphism of the gene for dopamine β-hydroxylase (DBH), which converts dopamine (DA) into norepinephrine, has been linked to psychostimulant abuse and psychotic symptoms in humans (Weinshenker and Schroeder, 2007). DBH knockout mice have been studied as an animal model of this human polymorphism and of psychostimulant abuse. DBH knockout mice, which are deficient in norepinephrine, are hypersensitive to the behavioral effects of cocaine, amphetamine, and the D_2 agonist quinpirole (Schank et al., 2006; Weinshenker et al., 2002). This hypersensitivity was surprising, since DBH knockout mice have reduced basal levels of DA as well as reduced DA release after amphetamine challenge. An explanation for this apparent paradox was apparently provided by in vitro receptor binding studies that reported a higher percentage of striatal D_2 receptors in the high-affinity state in DBH knockouts in comparison with heterozygote controls, which was hypothesized to represent a compensatory response to low extracellular DA levels in the knockouts (Schank et al., 2006; Seeman et al., 2005).

The purpose of this study was to test in vivo, the hypothesis that DBH knockout mice have an increased percentage of D_2 receptors in the high-affinity state and to determine whether DBH knockout mice might provide a model for increases in high-affinity state D_2 receptors in vivo. For this purpose, we scanned DBH knockout mice with the D_2 agonist radioligand [^{11}C]MNPA. In addition, we performed in vitro binding experiments on striatal homogenates with the antagonist radioligand [^{3}H]methylspiperone to measure B_{max} values and the percentages of high- and low-affinity states of the D_2 receptor.

MATERIALS AND METHODS

Radioligand preparation

[^{11}C]MNPA was prepared as previously described (Steiger et al., 2009). The specific activity of [^{11}C]MNPA at the time of injection was 82 ± 24 GBq/μmol (n = 8, syntheses). Chemical purity was >98%, radiochemical purity was >95%, and mean injected activity was 13 ± 6 MBq, which was accompanied by 0.17 ± 0.09 nmol of carrier.

Animals

DBH knockout mice and heterozygous controls (31 ± 5 g) were reared as previously described (Thomas et al., 1998; Weinshenker et al., 2002). We used heterozygous animals as controls as they are indistinguishable from wild-type mice, have normal levels of catecholamines and have been used as controls in prior studies (Thomas et al., 1998; Weinshenker et al., 2002).

PET studies

A total of 20 mice were imaged: 10 heterozygous and 10 knockout mice, with each group containing three females and seven males. PET scans were performed on the Advanced Technology Laboratory Animal Scanner (Seidel et al., 2003). Images were acquired and data analyzed with a reference tissue model as previously described (Ichise et al., 2003; Seneca et al., 2008). The outcome measure was binding potential (BP_{ND}), which is the ratio at equilibrium of specific binding to nondisplaceable uptake. The cerebellum was used as the reference region.

In vitro radioligand binding

Membrane homogenates were prepared from dissected striata of DBH knockout and heterozygous mice. Three to four striata from two mice were pooled per experiment (a total of five mice of each genotype) and binding was performed as previously described (Skinbjerg et al., 2009). In brief, for competition assays, membranes were incubated in binding buffer containing 0.2 mM sodium metabisulfite, 50 nM ketanserin, ~0.2 nM [^{3}H]methylspiperone (85.5 Ci/mmol, Perkin Elmer Life and Analytical Science, MA), and increasing concentrations of DA with and without the addition of 100 μM GTP. For saturation binding experiments, membranes were incubated with increasing concentrations (~0.02–2 nM) of [^{3}H]methylspiperone. The total receptor density was measured as B_{max} expressed as fmol/mg protein.

RESULTS

Following the i.v. injection of the agonist [^{11}C]MNPA, the radioactivity was concentrated in the striatum and had a similar time course in DBH knockout and heterozygous mice (Fig. 1A). Using the cerebellum as a reference region, the ratio at equilibrium of specific binding to nondisplaceable uptake (BP_{ND}) was not significantly different (BP_{ND} = 0.97 ± 0.06 and 1.06 ± 0.09 for heterozygous and knockout mice respectively, P = 0.866, n = 10 of each genotype) in these two groups of mice (Fig. 1B).

To separately measure receptor density (B_{max}) and radioligand affinity (K_D), we performed in vitro binding studies with the antagonist [^{3}H]methylspiperone. Both B_{max} and K_D values were not significantly different between DBH knockout and heterozygous mice. For knockout mice, the average B_{max} = 164 ± 31 fmol/mg and the average K_D = 0.39 ± 0.21 nM measured in striatal membranes from five animals. For heterozygous mice, the average B_{max} = 148 ± 30 fmol/mg and the average K_D = 0.33 ± 0.19 nM, measured in striatal membranes from five animals. Both the B_{max} (P = 0.72) and K_D (P =

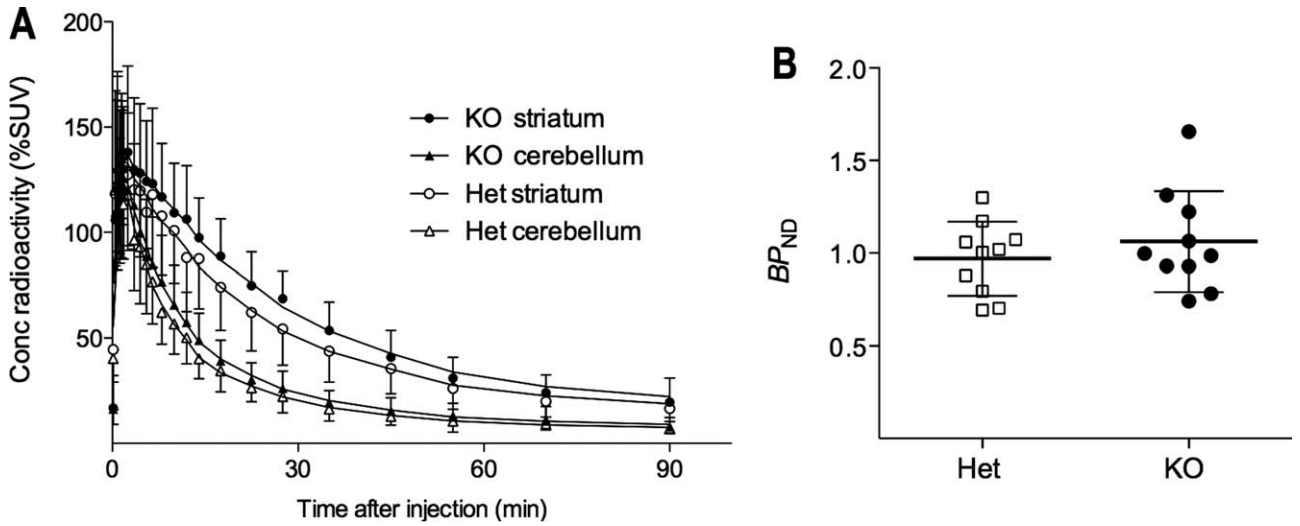

Fig. 1. (**A**) Time-activity curves for [^{11}C]MNPA and (**B**) binding potential (BP_{ND}) in DBH knockout and heterozygous control mice. BP_{ND} was insignificantly different between knockout (1.07 ± 0.3) and heterozygous controls (0.98 ± 0.2; mean ± SD, with 10 mice in each group).

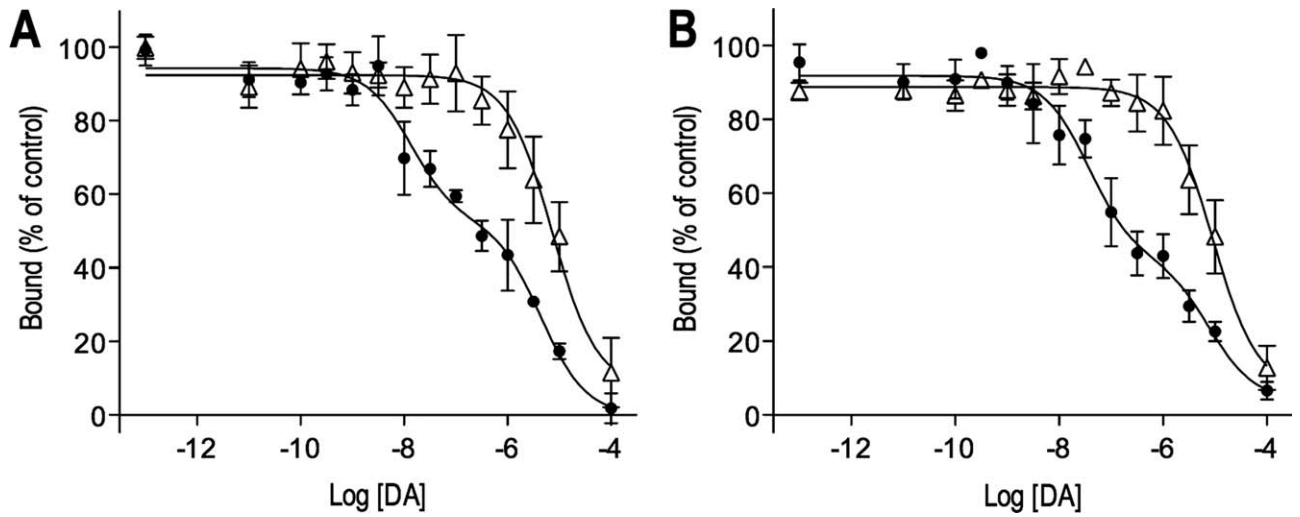

Fig. 2. Competition binding experiments on striatal membrane homogenates with [^{3}H]methylspiperone and dopamine in the absence (●) and presence (△) of guanine triphosphate (GTP). High-affinity agonist binding was observed in the absence of GTP, but the proportion of high-affinity binding was similar in heterozygous (**A**) and DBH knockout (**B**) mice. In the presence of GTP, one low-affinity binding site was observed. All experiments were performed in triplicate and repeated three times. K_I values and proportion of high affinity agonist binding are shown in Table I.

0.82) values were not significantly different between groups.

To determine the percentage of D_2 receptors in the high-affinity state, we performed competition binding studies with the antagonist [^{3}H]methylspiperone and DA, in the absence and presence of GTP. In the absence of GTP, the percentage of high-affinity receptors was similar in DBH knockout and heterozygous mice (56 ± 8% vs. 44 ± 6%, respectively, $P = 0.23$). In the presence of GTP, competition binding showed only low-affinity binding for both knockout and heterozygous mice (Fig. 2 and Table I).

TABLE I. Competition binding with [^{3}H]methylspiperone and dopamine in the presence and absence of GTP

Mouse strain	Ligand	K_{Ilow} (nM)	K_{Ihigh} (nM)	%High affinity
Heterozygote	DA	2990 ± 420	15.7 ± 8.9	44 ± 6%
	DA + GTP	4030 ± 1400		
Knockout	DA	4300 ± 670	17.0 ± 8.3	56 ± 8%
	DA + GTP	5250 ± 1900		

Values are mean ± SD from three experiments performed in triplicate.

DISCUSSION

The major finding of this study is that DBH knockout mice have the same density of D_2 receptors in the

high-affinity state as heterozygous controls, based on both in vivo and in vitro measurements. The in vivo uptake of the agonist radioligand [^{11}C]MNPA, which is a reflection of both receptor density and radioligand affinity, was similar in the two groups of animals. In addition, in vitro measurements using the antagonist radioligand [^{3}H]methylspiperone showed that the density, affinity, and proportion of receptors in the high-affinity state were not significantly different between these two groups.

Did endogenous DA block the in vivo radioligand binding of [^{11}C]MNPA and artifactually produce these results? Notably, microdialysis studies reported that DBH knockout mice have significantly reduced basal levels of DA in striatum (~66% of control) (Schank et al., 2006). As PET radioligands are sensitive to in vivo competition of endogenous DA, reduced basal levels of DA would be expected to increase BP_{ND} in DBH knockout mice. However, the BP_{ND} of DBH knockout mice was not different from that of control animals. In addition, a number of recent publications suggested that only one affinity state of D_2 receptor is detectable in vivo (Finnema et al., 2009; McCormick et al., 2008, 2009). As suggested by Finnema and colleagues, the in vivo receptor is presumably in the high-affinity state, because it is able to bind radioligand that is present at low (nanomolar to subnanomolar) concentrations.

Two potential confounding factors for PET imaging of small animals, such as mice, are partial volume effect (because of the small size of the target) and the radioligand occupying a significant percentage of receptors (because of the small number of receptors). As a result of limited spatial resolution of the PET camera, partial volume effects blunt the actual values of BP_{ND}. Ex vivo experiments in mice with the radioligand [^{3}H]NPA, which is pharmacologically very similar to [^{11}C]MNPA, reported BP_{ND} values of ~2.5 for striatum (Cumming et al., 2002), suggesting that our in vivo BP_{ND} values of ~1 were blunted by an expected 2.5-fold. These partial volume errors are assumed to be equal for heterozygous and knockout mice and would not have artifactually induced differences between the two groups. However, such partial volume errors would increase the magnitude of the difference necessary to be detected with in vivo imaging.

In addition to partial volume effects, the injected mass of radioligand in small animals may occupy a high percentage of receptors that violate the assumptions of tracer kinetic modeling. A rough estimate of receptor occupancy by [^{11}C]MNPA can be made by dividing the maximum specific binding in striatum for [^{11}C]MNPA (~2.8 nM) with reported B_{max} values (~25 nM) of rat striatum (Malmberg et al., 1996). This estimation would result in ~11% occupancy (2.8/25) of striatal D_2 dopamine receptors, thus on the high side for accuracy of tracer kinetic modeling. In addition, agonists are thought to bind to a subset of D_2 receptors in the high-affinity state, which would yield a greater than 10% occupancy by [^{11}C]MNPA. Nevertheless, the injected mass doses of radioligand were the same for knockout and controls and would not artifactually induce differences between the two groups. As mentioned for partial volume errors, the relative high mass dose will increase the magnitude of the differences necessary to be detected with in vivo imaging.

In addition to in vivo PET imaging, we also performed in vitro binding studies to measure the density and the percentage of D_2 dopamine receptors in the high-affinity state. In agreement with previous studies (Schank et al., 2006), we found no difference in the level of D_2 receptor expression (B_{max}) between DBH knockout and control mice. However, two prior studies reported that the percentage of D_2 receptors in the high-affinity state was increased in DBH knockout mice, although its statistical significance was not indicated (Schank et al., 2006; Seeman et al., 2005). In contrast, we did not find a statistically significant difference in the number of D_2 receptors in the high-affinity state between the DBH knockout and control mice. The reasons for these discrepant results are not clear, although they may be related to differences in methodology between the studies. In Schank et al. (2006) and Seeman et al. (2005), different approaches for detecting the high-affinity state were used: [^{3}H]raclopride binding in the presence and absence of GTP (which presumably caused endogenous DA to dissociate from the receptor leading to increased antagonist binding), as well as DA/[^{3}H]raclopride and DA/[^{3}H]domperidone competition assays. In contrast, we performed DA/[^{3}H]methylspiperone competition assays coupled with computerized curve fitting to quantitate the high- and low-affinity states of the receptor in washed membrane preparations. Given this, we reasoned that an overall better approach would be to use an agonist radioligand, and to perform in vivo assessments, since the agonist is thought to bind preferentially to the high-affinity state. Our current PET imaging results with [^{11}C]MNPA indeed support the notion that there is no increase in the high-affinity state of the D_2 receptor in DBH knockout mice.

In summary, we found no significant differences of D_2 receptors in the high-affinity state between DBH knockout and control mice with either in vivo PET scanning or in vitro binding experiments. Our results do not support previous in vitro data and indicate that DBH knockout mice have normal densities of D_2 dopamine receptors in high-affinity state, suggesting that other mechanisms likely underlie their behavioral hypersensitivity to psychostimulants.

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